

RESEARCH PAPER

Efficient acclimation of the chloroplast antioxidant defence of *Arabidopsis thaliana* leaves in response to a 10- or 100-fold light increment and the possible involvement of retrograde signals

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Abstract

Chloroplasts are equipped with a nuclear-encoded antioxidant defence system the components of which are usually expressed at high transcript and activity levels. To significantly challenge the chloroplast antioxidant system, *Arabidopsis thaliana* plants, acclimated to extremely low light slightly above the light compensation point or to normal growth chamber light, were moved to high light corresponding to a 100- and 10-fold light jump, for 6 h and 24 h in order to observe the responses of the water–water cycle at the transcript, protein, enzyme activity, and metabolite levels. The plants coped efficiently with the high light regime and the photoinhibition was fully reversible. Reactive oxygen species (ROS), glutathione and ascorbate levels as well as redox states, respectively, revealed no particular oxidative stress in low-light-acclimated plants transferred to 100-fold excess light. Strong regulation of the water–water cycle enzymes at the transcript level was only partly reflected at the protein and activity levels. In general, low light plants had higher stromal (sAPX) and thylakoid ascorbate peroxidase (tAPX), dehydroascorbate reductase (DHAR), and CuZn superoxide dismutase (CuZnSOD) protein contents than normal light-grown plants. Mutants defective in components relevant for retrograde signalling, namely *stn7*, *ex1*, *tpt1*, and a mutant expressing *E. coli* catalase in the chloroplast showed unaltered transcriptional responses of water–water cycle enzymes. These findings, together with the response of marker transcripts, indicate that abscisic acid is not involved and that the plastoquinone redox state and reactive oxygen species do not play a major role in regulating the transcriptional response at $t=6$ h, while other marker transcripts suggest a major role for reductive power, metabolites, and lipids as signals for the response of the water–water cycle.

Key words: Ascorbate peroxidase, light acclimation, photosynthesis, retrograde signalling, transcript regulation.

Introduction

In green plants, more than 97% of plastid proteins are encoded in the nucleus while the remaining fraction is encoded in the plastid genome (Abdallah *et al.*, 2000). Therefore, the co-ordination of plastid functions, in particular, of photosynthetic activity with complementary gene expression in the nucleus, relies on compartment-bridging

Abbreviations: AA, ascorbic acid; ABA, abscisic acid; AOX, alternative oxidase; APX, ascorbate peroxidase; DCMU, 3-(3,4-dichlorophenyl)-1, 1-dimethylurea; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; EEE, excess excitation energy; ex1, executor 1; GR, glutathione reductase; GSH/GSSG, reduced/oxidized glutathione, respectively; H, high light; L, low light; LOX, lipoxygenase; MDH, malate dehydrogenase; MDHAR, monodehydroascorbate reductase; N, normal light; PRX, peroxiredoxin; PS, photosystem; sAPX, stromal ascorbate peroxidase; SOD, superoxide dismutase; tAPX, thylakoid-bound ascorbate peroxidase; tpt, triosephosphate translocator; wwc, water–water cycle.

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communication systems (Baier and Dietz, 2005; Pesaresi *et al.*, 2007; Woodson and Chory, 2008). The information transfer from organelles to the nucleus, also called retrograde signalling, targets many processes outside the organelle including nuclear transcription and cytoplasmic translation (Escoubas *et al.*, 1995; Mussnug *et al.*, 2005).

The generation of reactive oxygen species (ROS) is part of photosynthetic metabolism. ROS accumulation is under tight homeostatic control by antioxidant enzymes and non-enzymatic antioxidants which decompose ROS. Above compatible low levels, ROS cause the oxidation of cell constituents and, therefore, metabolic imbalances and, eventually, damage to cell structures and cell death (Foyer *et al.*, 2009). Since all antioxidant enzymes are nuclear-encoded, retrograde signalling mechanisms control their expression and their activity (Mittler *et al.*, 2004; Baier and Dietz, 2005). Thus the regulation of the water–water cycle (wvc) is a good experimental system to reveal the mutual interaction of retrograde and anterograde signals between chloroplast and nucleus/cytoplasm. The superoxide anion radical O_2^- generated by the photosynthetic electron transport chain is rapidly dismutated to H_2O_2 and O_2 by the chloroplast superoxide dismutase isozymes CuZnSOD and FeSOD. Subsequent H_2O_2 reduction proceeds via two pathways. The ascorbate-dependent wvc implicates thylakoid-bound and stromal ascorbate peroxidase (tAPX, sAPX) coupled to monodehydroascorbate reductase (MDHAR), or dehydroascorbate reductase (DHAR), and glutathione reductase (GR; Asada, 1999). In addition, the ascorbate-independent wvc uses peroxiredoxins (Prx) as catalysts that use thiol-based peroxide-detoxification mechanisms. Three types of Prx operate in the chloroplast as well as glutathione peroxidases that have been shown to function as thioredoxin-dependent peroxidases like peroxiredoxins (Dietz, 2011; Navrot *et al.*, 2006; Koh *et al.*, 2007). Prx in *A. thaliana* chloroplasts are represented by four proteins, namely the highly similar 2-CysPrxA and 2-CysPrxB, as well as PrxQ and PrxIIIE (Horling *et al.*, 2003; Muthuramalingam *et al.*, 2009) and two glutathione peroxidases (Navrot *et al.*, 2006).

The chloroplast antioxidant enzymes are expressed at high levels under normal growth conditions and often respond moderately to changing light (Yoshimura *et al.*, 2000). *A. thaliana vtc1* mutants that accumulate only about 30% of wild-type ascorbate express unaltered sAPX and tAPX activity (Veljovic-Jovanovic *et al.*, 2001). Contrasting regulation occurs in response to stresses and in transgenic plants compromised in certain antioxidant systems (Mittler *et al.*, 2004). Over-expression of tAPX in tobacco increases tolerance of the transgenic plants to oxidative stress (Yabuta *et al.*, 2002). The authors concluded that tAPX activity might be a limiting factor in the antioxidative system under photo-oxidative stress in tobacco chloroplasts. The unaltered growth behaviour of *sapx/tapx* knock-out *A. thaliana* plants at various light intensities implies that this conclusion should not be generalized (Kangasjärvi *et al.*, 2008). Recent findings suggest that chloroplastic APX are not only responsible for photoprotection but also affect the cellular redox-state at the glutathione and ascorbate levels and thereby alter expression

of the H_2O_2 -sensitive genes (Maruta *et al.*, 2010). In line with these results, older leaves in particular of *sapx/tapx/vtc2* triple mutants are not able to detoxify H_2O_2 efficiently (Giacomelli *et al.*, 2007). Light shift experiments have been used to challenge photosynthetic metabolism and the antioxidant defence system, and have given significant insight into retrograde and systemic signalling processes. In pioneering experiments, Karpinski *et al.* (1997) exposed *A. thaliana* plants grown at $200 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ to $2000 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$. The fastest response to the 10-fold light increase is the up-regulation of bundle sheath associated ascorbate peroxidase 2 (APX2) transcripts after 7 min. Also the redox state of glutathione dropped quite rapidly from about 97% to 86% after 60 min of high light. In subsequent studies, bundle sheath-originated H_2O_2 was shown to be involved in systemic signalling from leaves exposed to excess excitation energy (EEE) to shaded leaves. The light regime included a jump from 200 to $2700 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (Karpinski *et al.*, 1999). Whole plant imaging technology based on the expression of the luciferase reporter under the control of the APX2-promoter allowed the spreading of the systemic signal to be monitored in this work. The application of exogenous abscisic acid (ABA) not only leads to increased cellular H_2O_2 level (Jiang and Zhang, 2003) but also induces APX2 expression even under low light intensities (Fryer *et al.*, 2003). In microarray experiments, the comparison of ABA- and H_2O_2 -treated plants disclosed that one-third of the transcripts are co-regulated by both effectors (Wang *et al.*, 2006). Since ABA also accumulates under high light treatment (Rossel *et al.*, 2006) the results suggest a central role for ABA in signalling events to high light-responsive gene expression. However, Galvez-Valdivieso *et al.* (2009) postulated that the initiation of ABA-biosynthesis under high light requires a decline in leaf water content and is not based on light-associated signals.

Koussevitzky *et al.* (2007) suggested that ABI4 has a central role downstream of the possible master switch GUN1 (Richly *et al.*, 2003). GUN1 integrates several retrograde signalling pathways from the chloroplast to the nucleus, namely the pathways transmitting signals linked to Mg protochlorophyllide IX, photosynthetic electron transport, oxidative stress and, in addition, to sucrose. This central role appears restricted to the early stages of seedling development. On the other hand, several signalling molecules have been identified that participate in operational control by adjusting nuclear gene expression to the demand of the photosynthesizing chloroplast (Baier and Dietz, 2005; Pesaresi *et al.*, 2006; Kleine *et al.*, 2009; Galvez-Valdivieso and Mullineaux, 2010).

This work addresses the regulation of the chloroplast antioxidant defence system in response to a challenging light shift of 10- and 100-fold photon flux density increases, respectively, to the same light regime of $800 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$. This study addresses the following topics. (i) Is the response of the wvc enzymes co-ordinate or separate? (ii) How strict is the coupling between the different molecular levels of response, that is, the induction of gene expression, transcript accumulation, protein accumulation, enzyme

activity, and metabolite levels? (iii) Are low-light-acclimated plants able to handle the extreme stress of a 100-fold light jump? It will be shown that the low light plants are extremely efficient in coping with such an extreme change in environmental conditions.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (wild type), the mutants *stn7* (Pesaresi *et al.*, 2009), *tpt* (Schneider *et al.*, 2002), *ex1* (Lee *et al.*, 2007), and *KatE* (Maurino and Flügge, 2008) were grown under controlled conditions (10 h light with 80 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$, and 21 °C/14 h dark and 18 °C, 50% relative humidity) on a 2:1:1 by vol. soil mixture of Fröhndorfer Erde Klocke P, perlite, and vermiculite. After 3 weeks of growing, plants were either transferred to 8 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (low light, L-light) or kept at 80 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (normal light, N-light) for another 10 d. Plants at the age of 4.5 weeks were transferred to high light (H-light, 800 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) 1 h after the onset of light. Control plants remained either under 8 or 80 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. At time points 0, 6, and 24 h H-light-treated and control samples were taken, and complete rosettes of 4–12 plants (N- and L-light-adapted plants, respectively) were harvested, immediately frozen in liquid nitrogen, and stored at –80 °C. For the combinatory experiment, effectors (ABA, DCMU, H-light) were administered to 5 mm diameter leaf discs that were punched out of leaves of 30-d-old control plants grown at 80 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. The discs floated upside-down on 0.1 mM CaCl_2 supplemented with 30 μM ABA, 10 μM DCMU, and 800 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ or all combinations of these three as indicated. The treatment was started 1 h after onset of light and lasted for 4 h prior to freezing the tissue in liquid nitrogen for further processing.

Production of KatE over-expressing A. thaliana

The genomic sequence from *E. coli* catalase (*KatE*; M55161) was amplified using Platinum Pfx DNA polymerase (Invitrogen, Karlsruhe, Germany) and cloned into pCR-Blunt II-TOPO (Invitrogen). The following primer combinations were used: *KatE* fow1 (5'-ACACCGGTCGCAACATAACGAAAAGAACCCA-3') and *KatE* rev1 (5'-ACGTCGACTCAGGCAGGAATTTTGTCAATCT-3'). To target *KatE* to the chloroplast a fragment containing the *tomato-RubisCO-small subunit* (*rbcS3C*; X66072) promoter (715 bp) and transit peptide (172 bp) was amplified by PCR using genomic DNA and the following primers: *rbcS3C* fow1 (5'-ACGAGCTCATCCAGAATTGGCGTTGGATTA-3') and *rbcS3C* rev (5'-AGCAACGGTGGAAGAGTCAGTTGCAACCGGTAT-3'). The fragment obtained was inserted upstream of the *KatE* coding region. To direct the expression in *A. thaliana*, the DNA encoding the plastidic precursor of *KatE* was cloned into a modified version of the binary vector pGreenII-nosBAR (Fahnenstich *et al.*, 2007) and transformation was conducted as described by Fahnenstich *et al.* (2007). Transformants were selected by resistance to BASTA and non-segregating T3 lines were produced. Leaf material was collected from selected plants and used for catalase activity measurements in 50 mM KH_2PO_4 , pH 7.0 as described by Havir and McHale (1987). The reaction was started by the addition of 10 mM H_2O_2 and followed at 240 nm at 25 °C. A line with 0.5-fold more activity than the wild type was chosen for this work.

Determination of mRNA levels

RNA isolation and the subsequent cDNA synthesis were performed according to Wormuth *et al.* (2006). Semi-quantitative RT-PCR

analysis was carried out as previously described (Finkemeier *et al.*, 2005). Supplementary Table S1 at JXB online lists the primer combinations used. PCR amplification was performed by 1 cycle at 94 °C for 3 min, and for the optimized number of cycles for each gene product for 45 s at 94 °C, 30 s at x °C, and 15 s at 72 °C, where x represents the primer-specific annealing temperature. Equal loading of each amplified cDNA was determined using the control Actin2 PCR product. Following separation of the PCR products on ethidium bromide-stained agarose gels, the transcript levels were estimated from densitometric readings of three independent experiments and expressed as relative expression ratios.

Quantitative real-time PCR (qRT-PCR) analysis was carried out on the iCycler™ Thermal Cycler (Bio-Rad, USA) with the iQ™ SYBR Green Supermix (Bio-Rad, USA) in a final volume of 20 μl according to the manufacturer's instructions. The iCycler was programmed to 95 °C for 1 min; 45×(95 °C for 30 s, 58 °C for 40 s, 72 °C for 45 s), 72 °C for 10 min followed by a melting curve programme (55–95 °C in increasing steps of 0.5 °C). Efficiencies of each reaction were calculated using LinRegPCR software (Ruijter *et al.*, 2009). Signal values were subsequently derived from the threshold cycles (average background subtracted) using the equation of Pfaffl (2001).

Antibodies

DHAR (At5g16710), and MDHAR (At1g6394) were cloned into pEXP5/NT-Topo vectors without putative signal peptides. Heterologous expression in *E. coli* and protein purification were performed as described previously (Laxa *et al.*, 2007). Purified recombinant protein was used to raise an antiserum against DHAR and MDHAR in rabbit (Pineda, Berlin, Germany).

SDS-PAGE and immunoblotting

Proteins were extracted in a buffer containing 50 mM TRIS-HCl (pH 6.8), 2% (w/v) CHAPS, and 0.1% SDS. Protein concentrations were determined using amido black 10B staining (Popov *et al.*, 1975; Schweikl *et al.*, 1989). SDS-PAGE and Western blot analyses were performed as described by Horling *et al.* (2002).

Chlorophyll a fluorescence measurements, determination of the electron transport rate and gas exchange

The steady-state quantum yield of PSII (F_v/F_m) was measured using the Mini-PAM Fluorometer (Walz, Germany) under the respective light condition as indicated. For determining recovery from photo-inhibition, plants were transferred to N-light conditions after the experiment and the potential quantum yield of PSII was determined again after 1 d and 5 d, respectively. Prior to light-dependent ETR measurements, plants were acclimated to darkness for 30 min and measured with a length of the actinic light periods of 2 min from low to high light. The data were used to calculate the electron transport rate by the equation: $\text{ETR} = \Phi\text{PSII} \times \text{PAR} \times 0.84 \times 0.5$ (according to manufacturer's instructions; Walz, Germany). The assimilation rate of N- and L-plants under high light was analysed by using the Arabidopsis pot holder of the portable gas exchange fluorescence System GFS-3000 according to the manufacturer's instructions (Walz, Germany). The measurements were conducted under constant gas flow rate (750 $\mu\text{mol s}^{-1}$), temperature (23 °C), relative humidity (55%), and light intensity (800 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) by utilizing atmospheric CO_2 .

Determination of chlorophyll and antioxidant contents

Chlorophyll extraction and calculation were performed according to Porra (2002). Reduced (AA) and oxidized ascorbic acid (DHA) were determined as described by Horling *et al.* (2003). Contents of glutathione were determined with an enzyme cycling assay based on sequential oxidation of GSH by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and reduction by NADPH in the presence of

glutathione reductase (Griffith, 1980) with few modifications. 200 mg frozen plant material was extracted in 1 ml ice-cold mix of 0.1 M HCl and 0.1 mM EDTA. The neutralized supernatant was used for the assay of GSH and GSSG. For total GSH content, 200 μ l of the supernatant was first incubated with 2 mM DTNB for 5 min followed by 15 min incubation with 5 μ l 2-vinylpyridine. After centrifugation, the resulting supernatant was used for the determination of the total GSH content. The reaction was started by the addition of glutathione reductase and changes in 5'-thio-2-nitrobenzoic acid absorbance were monitored at 412 nm for 8 min. To determine the GSSG content, the neutralized supernatant was incubated first with 2-vinylpyridine for 15 min followed by 5 min DTNB incubation. The difference between total glutathione and GSSG contents is presented as the GSH content.

Biochemical analyses of enzyme activities

Ascorbate peroxidases (APx) activity was measured by monitoring the decrease in absorbance at 290 nm according to Hossain and Asada (1984a). Dehydroascorbate reductase (DHAR) and monodehydroascorbate (MDHAR) were quantified as described by Hossain and Asada (1984b) and Hossain *et al.* (1984), respectively.

Hydrogen peroxide quantitation analysis

Leaves were frozen in liquid nitrogen. Hydrogen peroxide was quantified by chemiluminescence with luminol as described by Pérez and Rubio (2006).

Chloroplast ultrastructure analysis

Embedding, ultrasection preparation, contrasting and electron microscopic analysis were performed as described in Heiber *et al.* (2007).

Results

The comparison of 10- and 100-fold increases in photon flux density over growth intensity was chosen to challenge the antioxidant defence system of the chloroplast because this treatment gave reliable results of efficient regulation in response to an extreme challenge. *A. thaliana* plants grown in 8 μ mol quanta $\text{m}^{-2} \text{s}^{-1}$ light intensity which is slightly above the light compensation point grew very slowly during the 10 d period of low light acclimation (L-plants) and had small leaves with long petioles (Fig. 1A) while the plants at 80 μ mol quanta $\text{m}^{-2} \text{s}^{-1}$ (normal light, N-plants) developed normal rosette leaves. During the 10 d period of low light acclimation, the total leaf area had more than doubled, while that of normal light plants had increased about 8-fold (Fig. 1B). Chlorophyll *a* fluorescence measurements and ultrastructure analysis demonstrated that old and young leaves from L-plants were shade acclimated with low light

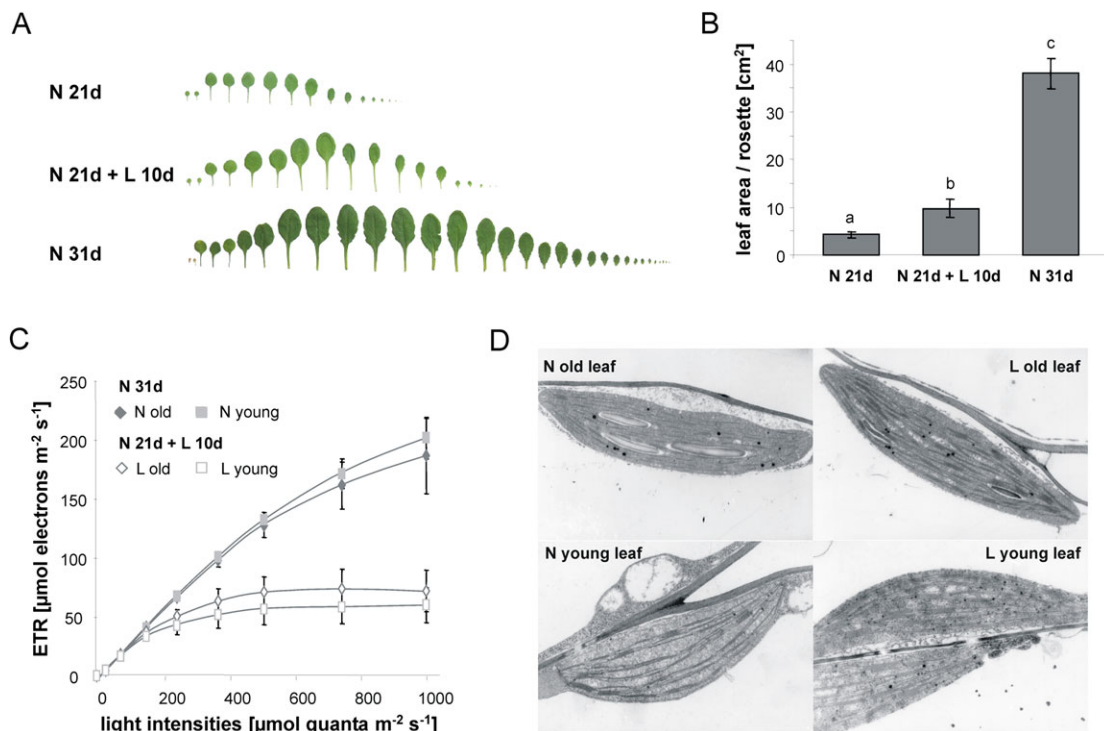


Fig. 1. Development and physiological light response of plants grown under low and normal light conditions. (A) Photographic documentation of fully dissected rosettes and (B) determination of leaf area (cm^2) per rosette of plants growing for 21 d under N-light conditions (80 μ mol quanta $\text{m}^{-2} \text{s}^{-1}$, N 21 d) and either transferred for 10 d to L-light (8 μ mol quanta $\text{m}^{-2} \text{s}^{-1}$, N 21 d+L 10 d, subsequently labelled as L-plants) or stayed for another 10 d under N-light (N 31 d, subsequently labelled as N-plants); $n=9$ from three independent experiments, means \pm SD, data groups of significant difference were calculated by Student's *t* test and are labelled with different letters ($P \leq 0.01$). (C) Light intensity-dependency of PSII electron transport rate (ETR, μ mol electrons $\text{m}^{-2} \text{s}^{-1}$) of newly developed leaves (young) and old leaves of N-plants (N 31 d) and L-light-adapted ones (N 21 d+L 10 d); $n=9$ from three independent experiments, means \pm SD, data groups of significant difference were calculated by Student's *t* test. No significant difference could be detected between young and old leaves in either N- or L-plants. (D) Ultrastructure of leaf chloroplasts in old and young N- and L-plants, respectively.

saturation (Fig. 1C) and chloroplasts contained many grana stacks (Fig. 1D), while electron transport (ETR) of old and young leaves from N-plants appeared not to be saturated at the highest light intensity used (Fig. 1C) and chloroplasts had many fewer grana stacks (Fig. 1D). It should be noted that the 2 min measuring period used for each light intensity might not have been sufficient to reach the steady state. These data show that the whole rosette was shade-acclimated at the end of the L-light acclimation period. At the age of 30 d, the plants were transferred to high light of $800 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (high light, H) 1 h after the regular light onset and samples were taken prior to transfer (0 h), and after 6 h and 24 h of treatment, respectively. Additional controls were taken from plants maintained at L- or N-light at $t=6$ h and $t=24$ h. The plants were continuously illuminated with their respective light intensity during the 24 h period. Thus the routine experiment discussed in the following consisted of 10 samples. The optimal time point for detailed analysis was searched by time-dependent analysis of mRNA levels of *sAPX* (Fig. 2). *sAPX* transcripts were unresponsive during the first 30 min following the transfer to H-light. It rapidly increased in N→H-plants and saturated by $t=3$ h, while it increased slowly in L→H-plants with saturation at around $t=6$ h. Thus 6 h appeared a good time point for the comparative analysis of the wwc enzymes at the transcript level. In addition, $t=24$ h was selected in order to investigate the consequences of transcriptional regulation at the protein level.

Chlorophyll contents in N-plants were approximately one-third higher than in L-plants. Although a small decrease in chlorophyll was observed in 24 h H-light which was not significant (Fig. 3A). The quantum yield of photosystem II at 6 h determined by chlorophyll *a* fluorescence analysis was high in all leaves maintained under the respective control conditions, but dropped by 18% in N→H(6 h) plants and by more than 30% in N→H(24 h), while the corresponding figures were 57% and 66% when analysing the L→H plants (Fig. 3B). To check for the severity and reversibility of photoinhibition, the plants were returned to N-light at the end of the H-light shift experiments. Photosynthetic quantum yield recovered during the following 24 h and returned to levels of unstressed plants

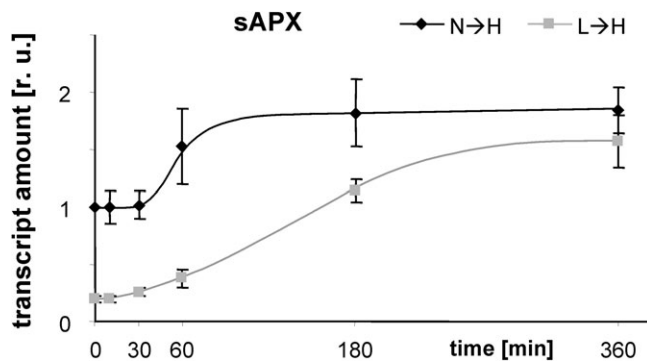


Fig. 2. Kinetics of transcriptional response of *sAPX* in N→H and L→H-plants. mRNA levels were quantified by qPCR in three experiments with two replicates. Data are means \pm SD.

after 5 d (Fig. 3C). To characterize the physiological state, protein- and RNA-levels were measured and revealed more than 50% lower protein and RNA amounts in L-plants (see Supplementary Fig. S1A, B at JXB online). For the

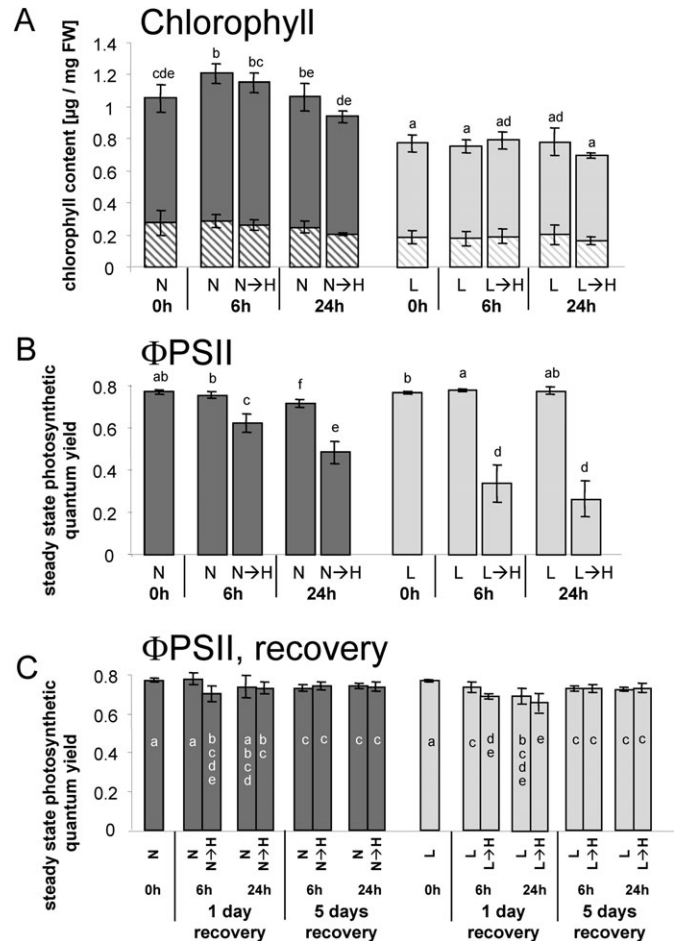


Fig. 3. Chlorophyll *a* and *b* contents and photosynthetic quantum yield (ΦPSII) during the high light transfer experiment and after recovery. (A) Chlorophyll *a* and *b* contents per fresh weight of L-, N-, L→H-, and N→H-plants at $t=6$ h and $t=24$ h and at the starting condition $t=0$: $n=10$ from three independent experiments, means \pm SD, data groups of significant difference were calculated by Student's *t* test and are labelled with different letters ($P \leq 0.05$). The ratios of chlorophyll *a* to *b* were not significantly different between the treatments. Chlorophyll *a*: solid, chlorophyll *b*: hatched. (B) Steady-state photosynthetic quantum yield of L-, N-, L→H-, and N→H-light plants at $t=6$ h and $t=24$ h and at the starting condition $t=0$ under the respective light condition. Chlorophyll *a* fluorescence transients were recorded with the pulse amplitude modulation technique and ΦPSII calculated. Data are means \pm SD of $n=30$ from three independent experiments, different letters mark significance groups by *t* test, $P \leq 0.01$. (C) Recovery of ΦPSII of L→H- and N→H-plants during a period of 1 d and 5 d in normal light. ΦPSII was measured in normal light: $n=30$ from three independent experiments, means \pm SD, different letters denote significance groups by *t* test, $P \leq 0.05$. Please note that the left and right hand side of this figure present measurements of the plants after 1 d and 5 d, respectively.

subsequent analysis, it is interesting that both N→H(24 h) and L→H(24 h) had significantly increased protein levels compared with 0 h and 6 h, and also that RNA amounts were tentatively higher. It should be noted that the L-plants developed thinner leaves. Thus leaves from N-plants had a specific leaf area of $213 \pm 17 \text{ g m}^{-2}$ compared with L-leaves with $148 \pm 20 \text{ g m}^{-2}$ according to this L-leaves had 31% less weight.

Glutathione and ascorbate levels were analysed in order to characterize the state of the two major low molecular mass antioxidant systems known to be linked to redox homeostasis and signalling which also function as main reductants of the wwc (Fig. 4A, B). Glutathione levels of L-plants determined on a fresh weight basis were about half that of N-plants, ascorbate levels were even slightly lower. Total glutathione levels of N-plants increased slightly upon H-light treatment and strongly in L→H-plants within 6 h (Fig. 4A). This amount was maintained during the 24 h

H-light treatment of L-plants, but further increased in N→H-plants to about 180% of the N-plant controls. The oxidation level of the glutathione pool ranged between 6.9% [N→H(6 h)] and 13.4% [L→H(24 h)]. Significant differences in glutathione oxidation state were not observed between the controls at 0, 6, and 24 h. Importantly, the glutathione pool was more oxidized neither in N→H(6 h)-plants compared with N(6 h)-plants nor in L→H(6 h)-plants compared with L(6 h)-plants. The only significant increase in glutathione oxidation was seen for L→H(24 h)-plants compared with L(24 h)-plants. The ascorbate plus dehydroascorbate (DHA) contents changed in parallel to the glutathione contents among the different treatments and time points (Fig. 4B). The proportion of (reduced) ascorbate ranged between 47% and 90%, the latter highly reduced state was seen in the N→H(24 h)-condition. H_2O_2 levels were similar in N→H at $t=6 \text{ h}$ compared with plants maintained in N-light, with an insignificant increase at $t=24 \text{ h}$

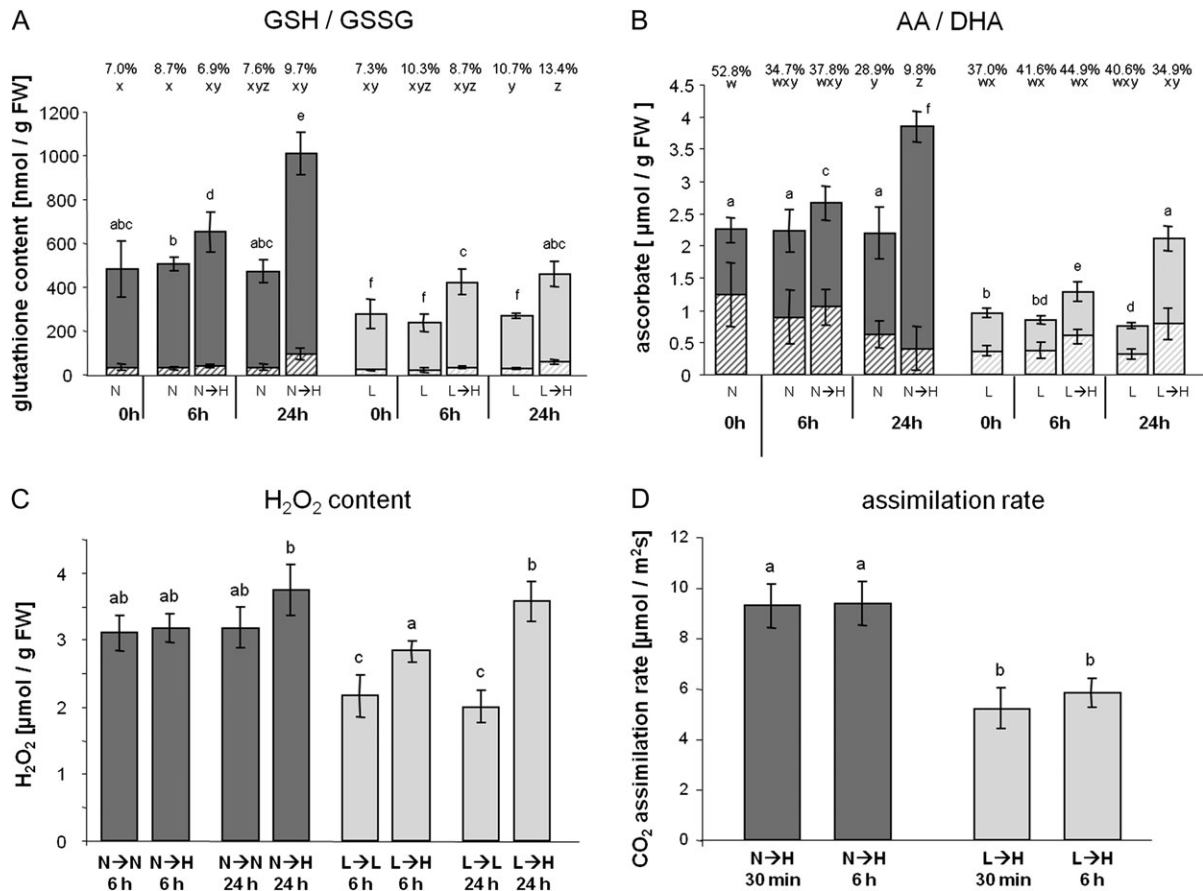


Fig. 4. Leaf contents of glutathione, ascorbate, and H_2O_2 and the assimilation rates in L-, N-, L→H-, and N→H-leaves at $t=6 \text{ h}$ and $t=24 \text{ h}$. (A) Contents of glutathione in its reduced (GSH; solid) and oxidized form (GSSG; hatched) at $t=6 \text{ h}$ and $t=24 \text{ h}$ and at the starting condition $t=0$: $n=6$ from three independent experiments with replicates, means \pm SD, data groups of significant difference were calculated by t test and are labelled with different letters ($P \leq 0.05$). The percentage values given above the columns present the oxidation state of the glutathione pool. (B) Ascorbate (solid) and dehydroascorbate contents (hatched) at $t=6 \text{ h}$ and $t=24 \text{ h}$ and at the starting condition $t=0$. $n=6$ from three independent experiments with replicates, means \pm SD, data groups of significant difference were calculated by t test and are labelled with different letters ($P \leq 0.05$). The percentage values given above the columns present the oxidation state of the ascorbate pool. (C) H_2O_2 levels in leaves at $t=6 \text{ h}$ and $t=24 \text{ h}$ were quantified by luminol assay as described in the Materials and methods. The data are mean \pm SD from three independent experiments. (D) CO_2 assimilation rate of L→H and N→H-plants at 30 min after transfer and after 6 h. Data are means of $n=3 \pm$ SD.

h. In L-plants H_2O_2 levels were 31% higher in high light compared with L-plants at $t=6$, and by 76% at $t=24$. However, neither after 6 h nor 24 h, were H_2O_2 levels in L-plants higher in L→H-plants than in N→H-plants (Fig. 4C). In addition, CO_2 assimilation rates were compared in L→H and N→H plants right after reaching the maximum after transfer at $t=30$ min and 6 h. Electron transport rates as also the carbon assimilation per leaf area were lower in L- than in N-plants, but maintained over the 6 h H-light treatment (Fig. 4D).

Protein amounts of APX isoforms, DHAR, MDHAR, GR, and SOD were assessed by Western blot analysis (see Supplementary Fig. S2 at *JXB* online). The antibody of Kangasjärvi *et al.* (2008) recognized the tAPX, sAPX, and cAPX isoforms which separate well in SDS-PAGE. Band intensities were quantified by gel scan analysis from three experiments and the protein data compared with the data from transcript quantification by qPCR (Fig. 5). cAPX protein, which represents a cytosolic isoform mix, was most responsive to H-light treatment and the increase was similar

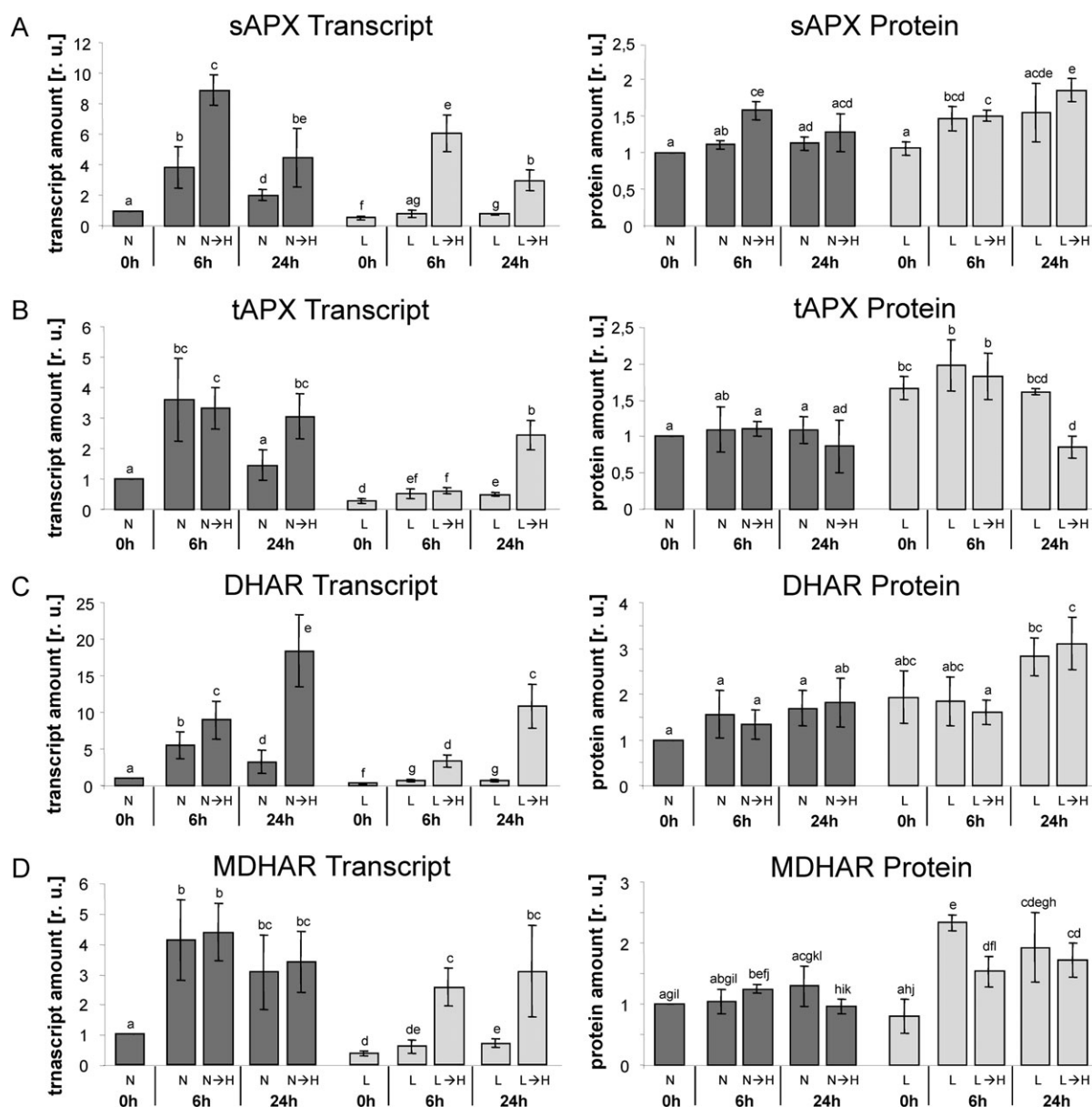


Fig. 5. Comparison of transcript and protein amounts of sAPX (A), tAPX (B), DHAR (C), MDHAR (D), GR (E), and CuZnSOD (F) in L-, N-, L→H-, and N→H-leaves at 0, 6, and 24 h. Band intensities were quantified densitometrically in three blots (as shown in Supplementary Fig. S2 at *JXB* online) by gel scan analysis and are expressed relative to RubisCO amounts quantified by Ponceau S staining on the nitrocellulose membranes following semidry transfer. Transcript amounts were quantified by qPCR and related to actin contents. qPCR data represent the average from three independent experiments with two technical replicates each. The significance of difference was calculated using Student's *t* test with $P \leq 0.05$ (transcript) and $P \leq 0.1$ (protein) and groups belonging to one significance group are labelled with same letters.

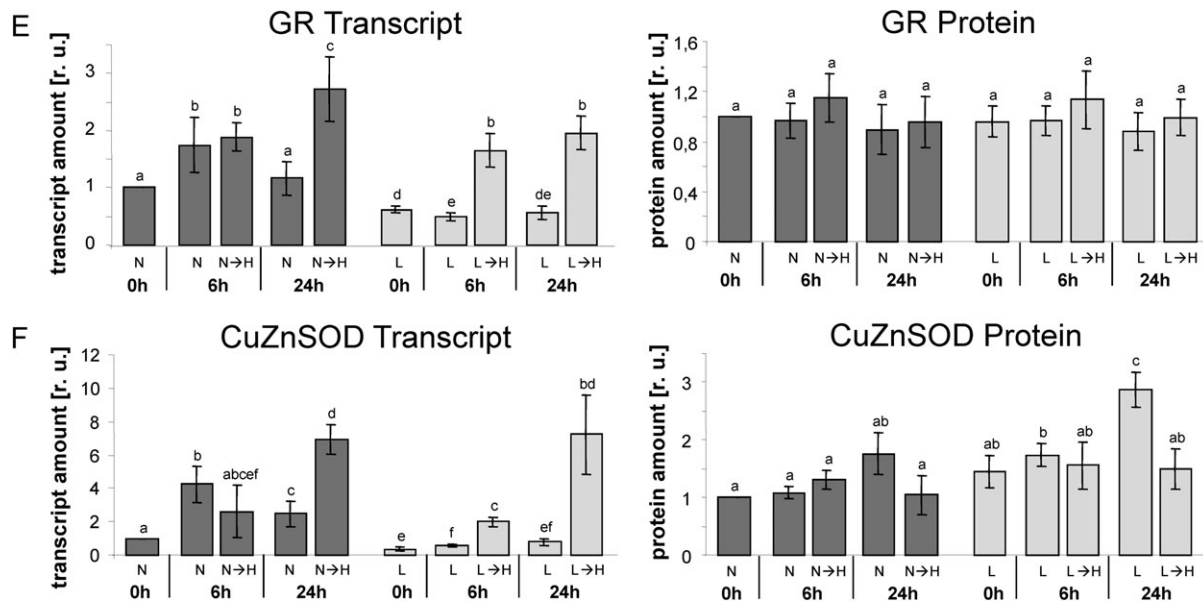


Fig. 5. Continued

at 6 h and 24 h (see [Supplementary Fig. S2 at JXB online](#)). sAPX and tAPX protein amounts revealed less regulation (Fig. 5A, B). On average, sAPX protein was similar in L(0 h) and N(0 h), and up-regulated in L(6 h), L→H(6 h), and N→H(6 h). tAPX like DHAR (Fig. 4C) and CuZnSOD (Fig. 5F) protein amounts were higher in L-plants by 50–120% compared with N-plants. tAPX protein was rather unresponsive to the treatments, except for the significant drop of tAPX protein amounts in L→H(24 h) (Fig. 5B). By contrast, transcript levels strongly varied between the different light intensities. All transcripts were lower at 0 h than at 6 h in N-plants which is more likely to be associated with photosynthetic activity than with circadian rhythm since the amplitudes of circadian oscillations of these transcripts appear to be smaller in experiments defining transcripts regulated by circadian rhythm than observed here (Bläsing *et al.*, 2005). sAPX transcript in N-plants increased between 0 h and 6 h and was further accumulated in N→H(6 h)-light. The strongest response of sAPX transcript was seen in L→H(6 h), with more than a 6-fold increase compared with L(6 h). The H light-dependent increase was less pronounced for L→H(24 h). For tAPX transcript, significant differences between N→H and N, as well as L→H and L were only seen at 24 h.

When comparing transcript regulation and protein amounts for DHAR (Fig. 5C), MDHAR (Fig. 5D), GR (Fig. 5E), and CuZnSOD (Fig. 5F), the following general and specific responses appear noteworthy. In N-plants, the MDHAR transcripts were unresponsive except for a low transcript level at the beginning of the experiments corresponding to 1 h after the end of the dark period (0 h). GR, SOD, and MDHAR transcripts were not increased in N→H(6 h) compared with N(6 h). All transcripts except MDHAR were higher in N→H(24 h) than in N(24 h). Low-light-acclimated plants upon transfer to H-light (6 h) revealed up-regulation for *sAPX*>*MDHAR*>*GR*>*D-*

HAR>*SOD*>*tAPX* in decreasing order of magnitude. This enhancement was more pronounced at 24 h for DHAR and SOD. One could assume that transcript regulation at 6 h might be reflected by complementary changes of protein levels and enzyme activities at 24 h, for example, for APX. However, such a dependency could not be observed (Fig. 6). APX activity was significantly increased in N→H(6 h), N→H(24 h), and L→H(24 h) by 35, 50, and 34%, respectively (Fig. 6A). This increase possibly reflects the increase in band intensities observed for cAPX in the Western blots (see [Supplementary Fig. S2 at JXB online](#)). Protein-related DHAR and MDHAR activities were higher in L-plants than in N-plants at 0, 6, and 24 h (Fig. 6B, C). The only strong increase was seen for DHAR activity in N→H(24 h) plants relative to N(24 h) to 176% of the control (Fig. 6B). From this analysis it is concluded that the strong transcript regulation taking place during the 10-fold and 100-fold light jump was sufficient and probably necessary to maintain the wwc enzyme proteins, or to bring them up to the adequate level in those cases where lower protein levels had been encountered as in the case of MDHAR protein.

Light-intensity-dependent regulation of nuclear gene expression depends on retrograde signalling. Literature evaluation resulted in transcripts that have been used or could be indicative of the involvement of specific signalling pathways in acclimation (Fig. 7). Sets of marker transcripts were selected to address signalling-related specific retrograde signals, i.e. to metabolites (sugar, *APL3*, and *β-AMY*) (Baier *et al.*, 2004), ABA (*RD29a* and *COR47*) (Sanchez *et al.*, 2004), lipid signalling (*LOX3*, *PPTE*) (Goda *et al.*, 2008), the plastoquinone redox state (*PETE2*, *LHCP2.3*) (Pfannschmidt *et al.*, 2001, Yang *et al.*, 2001), H₂O₂ (*FER1*, *PKRP*) (op den Camp *et al.*, 2003), singlet oxygen (*BAP1*, *NODL*) (op den Camp *et al.*, 2003), and excess reductive power (*AOX1a*, 2) (Yoshida and Noguchi, 2009). H₂O₂-

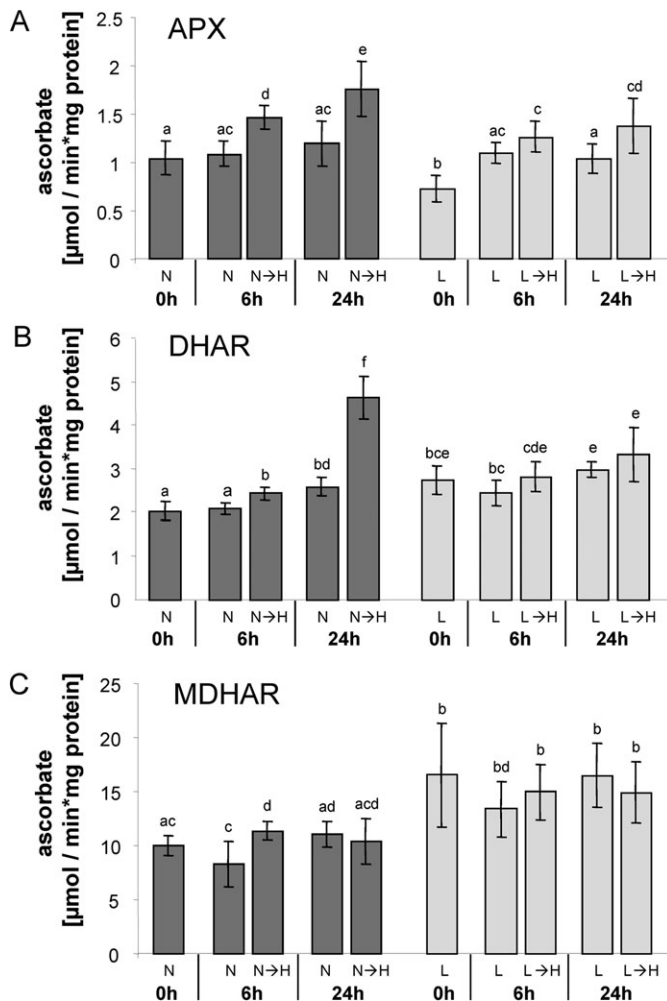


Fig. 6. Activities in total leaf extracts of total APX (A), DHAR (B), and MDHAR (C) in L-, N-, L→H-, and N→H-leaves at 0, 6, and 24 h. Data represent means \pm SD from three independent experiments with two replicates each. Student's *t* test with $P \leq 0.05$ was applied and groups belonging to one significance group are labelled with same letters.

markers *FER1* and *PKRP*, lipid marker *LOX3*, excess reductive power marker *AOX1*, and sugar marker *APL3* were differentially regulated both in N→H(6 h) and L→H(6 h) leaves compared with their respective controls with a somewhat enhanced response in formerly L-light-acclimated plants. $^1\text{O}_2$ -markers *BAP1* and *NODL* as well as *AOX2* gave no clear pattern and were mostly unresponsive. Transcript levels of ABA markers *RD29a* and *COR47* decreased in H-light. The PQ redox state marker PETE2 was hardly affected and LHCB2.3 decreased in H-light as expected as a response to excessive light. Bundle sheath *APX2* transcript was increased in N→H(6 h)-plants but not in L→H(6 h)-plants, and also responded stronger in N→H- than in L→H-plants at 24 h (Fig. 8).

A. thaliana mutants deficient in processes involved in retrograde signalling were tested for the response of wwc enzyme transcripts to the N→H(6 h)- and L→H(6 h)-light shift. STN7 mediates plastoquinone-dependent signalling in state transition and long-term response (Pesaresi

et al., 2009), EX1 mediates $^1\text{O}_2$ signals originating from excessively accumulated protochlorophyllide in the flu mutant (Lee *et al.*, 2007) and triosephosphate-phosphate translocator (TPT) provides the main route for the export of assimilates during the light (Schneider *et al.*, 2002). In addition, the expression of an *E. coli* catalase *KatE* in chloroplasts allowed us to investigate the involvement of reduced chloroplast-derived H_2O_2 in transcription regulation. Transcript pattern regulation after a light increase was similar in *stn7*, *ex1*, *tpt*, and *KatE* mutants as in the wild type (see Supplementary Fig. S3 at JXB online).

To address the role of combinatorial signals on *sAPX* expression leaf discs were floated upside-down on 0.1 mmol l^{-1} CaCl_2 supplemented with $30 \text{ } \mu\text{mol l}^{-1}$ ABA, $10 \text{ } \mu\text{mol l}^{-1}$ 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or exposed to $800 \text{ } \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$, or a combination of these. *RD29a* was taken as an established marker transcript responsive to ABA (not shown). DCMU suppressed *RD29a* accumulation while it was unresponsive to H-light. Each combination with ABA stimulated *RD29a* accumulation, however it was less pronounced than in the case of ABA (not shown). In a converse manner, *sAPX* was unresponsive to ABA, suppressed by DCMU, and stimulated by H-light. H-light stimulation was dampened or lost in all combinations, whereas the effect of DCMU treatment appeared predominant on suppressing the *sAPX*, underlining the conclusion from marker transcript analysis that reductive power might be a crucial signal in the *sAPX* response (Fig. 9).

Discussion

Reprogramming of metabolism and development upon transfer to high light

A. thaliana plants that had been acclimated to extremely low light intensities showed morphogenic differences of the shoot compared with N-plants. Petioles were elongated and leaf blades thinner and smaller than in N-light-grown plants. In addition, chlorophyll contents in L-plants were decreased compared with N-plants, even more pronounced on area-based chlorophyll contents due to the thinner leaf morphology, namely $22.0 \text{ } \mu\text{g cm}^{-2}$ in N-plants and $11.4 \text{ } \mu\text{g cm}^{-2}$ in L-plants. The area-based protein content was even decreased by 63% from $199 \text{ } \mu\text{g cm}^{-2}$ in N-leaves to $74 \text{ } \mu\text{g cm}^{-2}$ in L-leaves. These changes in morphology, chloroplast ultrastructure, and light-intensity-dependent photosynthetic electron transport rates indicate acclimation to shade, shade avoidance responses, and optimization of light harvesting in L-plants despite their metabolic state of carbohydrate and energy deficiency (Franklin and Quails, 2010). It should be noted that the spectral light quality during growth was similar in low, normal, and high light regimes. The results show that L-plants are remarkably flexible and efficient to cope with and acclimate to the excess light. Tissue damage was neither observed after 6h nor 24 h.

A contribution of light receptors such as phytochrome or cryptochrome to the acclimation response cannot be

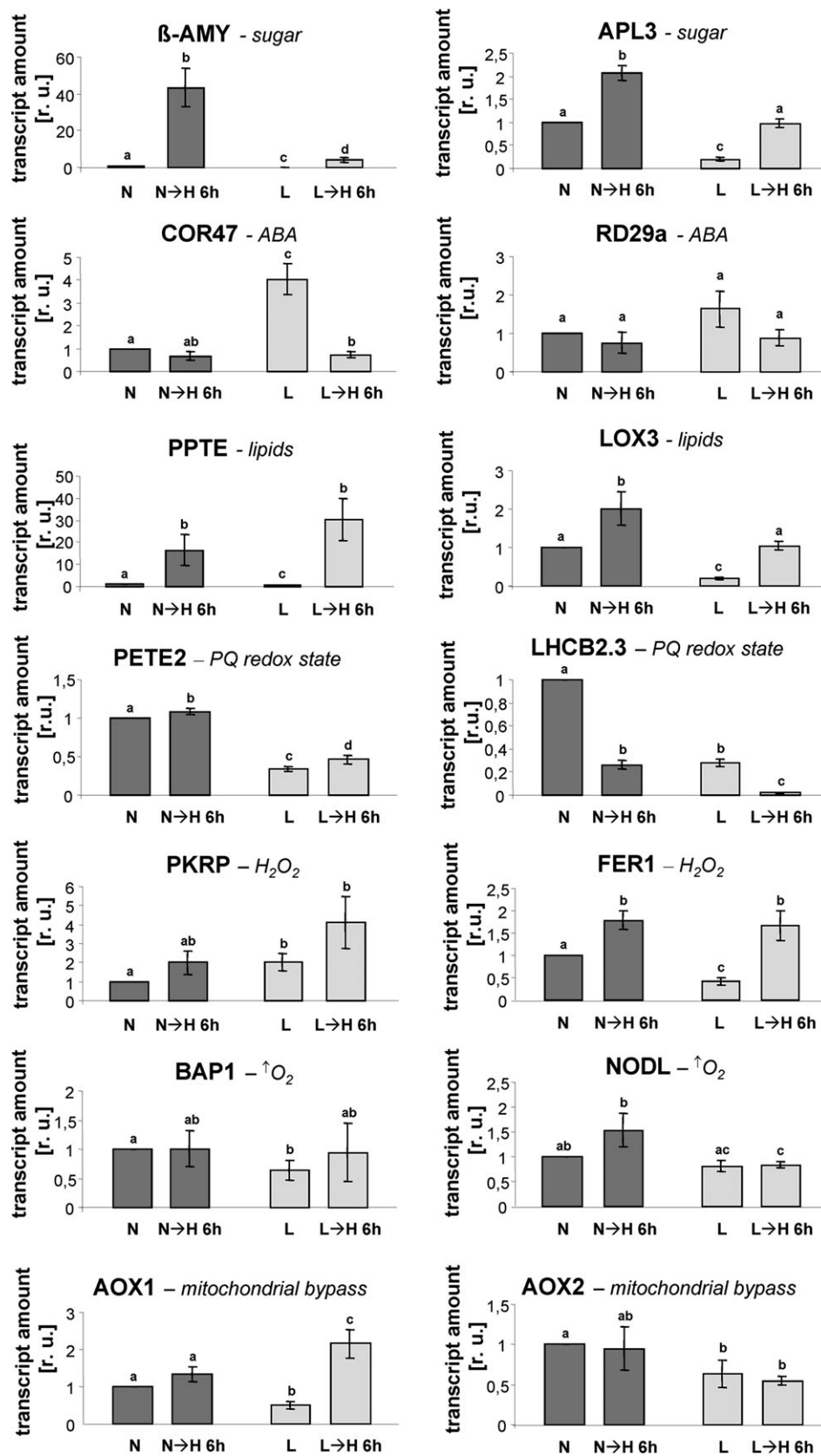


Fig. 7. Response of marker transcript amounts in L-, N-, L→H-, and N→H-leaves at 6 h. Transcript amounts were quantified by qPCR. Data are means ±SE from three experiments with duplicate determinations each. Actin2 was taken as reference transcript.

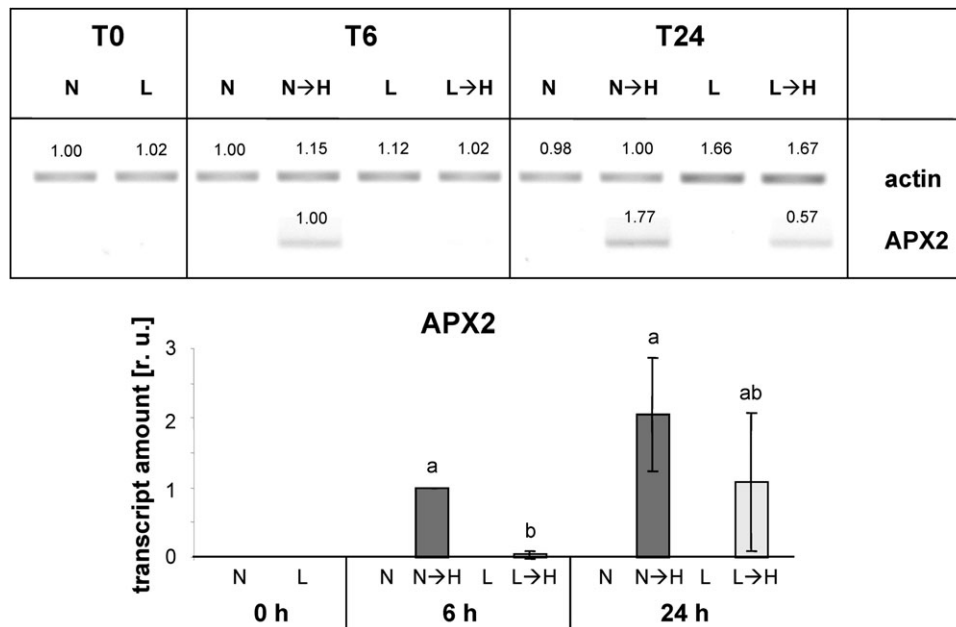


Fig. 8. Response of APX2 transcript in the light shift experiment. Band intensities were (semi-)quantified in three independent experiments and are shown as means \pm SD in the lower figure. Significance of difference was calculated using Student's *t* test and is marked with different letters.

excluded in the entire response but is unlikely to explain the regulation of the wwc enzymes and the antioxidant defence network due to the high similarity of the response in N→H(6 h) and in L→H(6 h). The Genevestigator database was queried for the effect of *cry* deficiency or specific treatments affecting phytochrome-dependent signalling such as the application of a low ratio of red to far red light (R/FR). The expression of enzymes of the ascorbate-dependent wwc was unaffected in the *phy*, *cry1*, and *hy5* mutants and by the R/FR-illumination (Genevestigator; Hruz *et al.*, 2008; Kleine *et al.*, 2007). Likewise, Fey *et al.* (2005) showed that defects in these light sensory systems do not alter the short-term response of the transcriptome to changing light quality preferentially exciting either PSII or PSI. It is concluded that cryptochrome and phytochrome are not involved in adjusting the antioxidant defence system of the chloroplast.

Following the transfer to H-light conditions, the transcriptome for wwc enzymes at 6 h was reorganized without changes in total protein and RNA contents (see [Supplementary Fig. S1](#) at *JXB* online). Only after 24 h, did both RNA and proteins reveal a trend to increase in H-light; in the L→H-plants by almost 100%. Thus the first 6 h were characterized by a profound reorganization of gene expression and protein composition without a significant change in total resource allocation.

Regulation of the water–water cycle enzymes as example of retrograde targets

Optimal expression and activity of the antioxidant system of the chloroplasts relies on gene expression regulation by retrograde signalling, since all its elements are encoded by

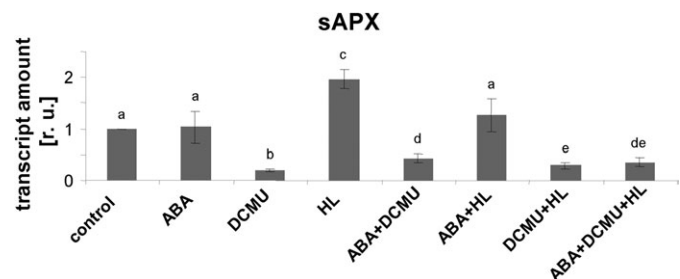


Fig. 9. Combinatorial effect of abscisic acid, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), high light, and combinations of these on sAPX transcript amounts. Leaf discs floated upside-down on 0.1 mM CaCl_2 (control) or supplemented with 30 μM ABA, 10 μM DCMU, and 800 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ or all combinations of these three. Transcripts were quantified by qPCR in three independent experiments. The data were related to transcript level of the housekeeping protein actin 2. The data are means \pm SD, $n=6$ from three different experiments.

the nuclear genome. However, the active and innervating regulatory pathways have not received major attention. Single or double deletions of sAPX and tAPX activities in *A. thaliana* are compensated by other antioxidant systems such as 2-CysPrx, enabling the plants to grow wild-type-like under non-stressed conditions (Kangasjärvi *et al.*, 2008), however, lack of tAPX leads to higher oxidized protein contents (Maruta *et al.*, 2010). The light shift treatment employed in this study induced strong transcript responses for enzymes of the wwc. The starting levels at 0 h as well as the 6 h and 24 h responses on the protein and transcript levels of the five enzymes involved in the wwc were different in each case. However, some general relationships can be found.

Protein levels differed among the treatments in the range between 1.5- (GR, sAPX), 2- (tAPX), and 3.5-fold (DHAR, MDHAR), and thus were significantly less responsive than transcript amounts. tAPX and CuZnSOD protein amounts even declined after 24 h H-light treatment indicating the high need and sensitivity of the thylakoid-bound scavenging system under H-light treatment and also the involvement of protein degradation that is probably enhanced in H-light. Chloroplast ascorbate peroxidases are sensitive to ROS-mediated inactivation when ascorbate levels fall below a critical threshold (Miyake and Asada, 1996). *A. thaliana* plants lacking tAPX accumulate more H₂O₂ and oxidized protein and show more severe changes in gene expression than plants lacking sAPX (Maruta *et al.*, 2010) suggesting that tAPX is particularly important for photoprotection and gene regulation under photooxidative stress.

The inverse behaviour of increasing tAPX and CuZnSOD mRNA and decreasing protein amounts in L→H(24 h) suggests that there exists a retrograde feedback mechanism between the thylakoid-associated ROS detoxification system and its own gene expression in the nucleus. Surprisingly, tAPX transcript was unresponsive to H-light at 6 h when compared with the respective control and only responded after 24 h when the tAPX protein amount had started to decline, indicating the action of some feedback regulation. By contrast, sAPX transcripts increased early enabling maintenance of constant sAPX protein amounts even at 24 h. Similarly, regulation of GR and MDHAR transcript levels appeared to maintain the respective proteins on a high level. GR and tAPX transcript levels changed 3-fold, while DHAR was most dynamic with a 60-fold difference between L(0 h)-plants and N→H(24 h)-plants. Also, the kinetics of response differed between the different wwc enzymes, with sAPX being rapidly responsive while tAPX only responded after 6 h H-light treatment. Interestingly, the H₂O₂-levels at N→H(24 h) and even more at L→H(24 h) were increased compared with the respective controls, indicating some sort of cumulative redox imbalance. However the H₂O₂-accumulation has to be seen in the context of the continuous H-light treatment for 24 h without a dark phase. The acclimation efficiency particularly of L-plants is impressive considering this extreme challenge.

The involvement of ROS in H-light response

Previous studies in which plants have been grown under low light and shifted to high light conditions, sometimes referred to as EEE treatment, indicated that ROS such as superoxide and hydrogen peroxide are involved in eliciting the transcriptional response (Karpinski *et al.*, 1997, 1999). These studies employed and established APX2 as an exemplary regulatory target being up-regulated at the transcript level upon EEE treatment. Subsequently, screenings were based on the expression of reporters linked to the APX2 promoter. ROS, glutathione, and ABA, each one derived from the bundle sheath, were identified as important components in the EEE acclimation programme. The *A. thaliana* mutant *regulator of APX2 1-1* (*rax1-1*)

constitutively over-expresses APX2 and carries a mutation in the γ -glutamylcysteine synthase (GSH1) gene (Ball *et al.*, 2004). Glutathione levels in *rax1-1* were in the range of about 30% of the wild type. Low antioxidant levels and specific additional effects of the mutated GSH1 allele were suggested to account for the constitutive up-regulation of APX2 which coincides with increased pathogen sensitivity. Interestingly, APX1, FeSOD, and MDHAR1 transcript and activity levels are up-regulated in *rax1-1*. However, in our study the low glutathione level in L-plants does not explain the response upon L→H-transfer, because L-plants (i) did not express APX2 under L-conditions, (ii) did not up-regulate APX2 transcript upon transfer to H-light (L→H(6 h)) (Fig. 8), and (iii) the ratio of glutathione to protein was quite similar in L- and N-plants suggesting that the glutathione concentration in plasmatic compartments such as stroma and cytosol was unchanged in L-plants. The whole leaf tissue H₂O₂ levels at *t*=6 h were identical in N-plants, N→H-plants, and L→H-plants, but lower in L-plants. Thus H₂O₂ could contribute to signalling at *t*=6 h in L-plants but not in N-plants. The overall response of the wwc enzyme transcriptome was qualitatively similar and quantitatively stronger in the L→H-plants. The transcripts that were suggested by op den Camp *et al.* (2003) as suitable H₂O₂ markers were up-regulated in H-light-treated plants. However, the pathways triggering the up-regulation of PKRP and FER1 transcripts in methylviologen treated cells (op den Camp *et al.*, 2003) are unknown and might involve redox imbalances independent of accumulating H₂O₂. In line with such an assumption, the *KatE* lines over-expressing an *E. coli* catalase in the chloroplast showed a wild-type-like response of the wwc enzymes during the treatment. Therefore, H₂O₂ released from the chloroplast is unlikely to be a major signal in controlling the H-light response of the cell. Similarly, the two transcripts selected as markers for singlet oxygen-dependent signalling (BAP1, NODL: op den Camp *et al.*, 2003), despite some heterogeneity in their response, support the view that singlet oxygen is also not a major signal in the regulation of wwc enzymes.

Redox, ABA, lipids, and metabolic signalling cues in L→H light acclimation of the wwc

Systemic signalling of excess excitation energy (Miller *et al.*, 2009), activation of APX2 expression (Bechthold *et al.*, 2008) and also ABA-triggered ROS-dependent stomatal closure involve the plasma membrane associated NADPH oxidases RBOHD and RBOHF (Zhang *et al.*, 2009). However, ABA appears not to be a major factor in signal transduction during light shift-dependent up-regulation of wwc enzymes. APX2 transcript was hardly up-regulated in L→H-plants (Fig. 8). As described above, APX2 is closely linked to H₂O₂ accumulation in bundle sheath cells (Karpinski *et al.*, 1997, 1999; Fryer *et al.*, 2003). Galvez-Valdivieso *et al.* (2009) concluded that APX2 transcript accumulation depends on linear electron transport, glutathione, ABA, and extracellular H₂O₂. But typical target transcripts of ABA stimulation, namely RD29a and

COR47 (Sanchez *et al.*, 2004), responded with down-regulation to H-light irrespective of the previous L- or N-light acclimation.

ABA functions in stress acclimation, stomatal closure, and retrograde signalling. In addition to the overall concentration of ABA as an effector of nuclear gene expression, that is, signalling linked to synthesis, degradation, and long-distance transport of ABA, also the ABA distribution between chloroplast and cytosol must be considered as a potential mechanism of retrograde signalling. ABA is a weak anion. The protonated form diffuses through membranes and the anion is trapped in alkaline compartments (Heilmann *et al.*, 1980). Increases in light intensities that acidify the thylakoid lumen and alkalize the stroma will withdraw ABA from the cytosol as suggested by kinetic modelling and simulation (Slovik and Hartung, 1992). The extreme L→H- and N→H-shifts performed here cause strong proton pumping from the stroma into the thylakoid lumen (Oja *et al.*, 1986). This would trap ABA in the anionic form in the chloroplast and deplete the cytosol as long as no net synthesis compensates for the redistribution reaction. An effect of increased ABA on *sAPX* expression can be excluded based on the lack of response of *sAPX* to ABA administration in leaf disc experiments (Fig. 9) clearly differentiating *sAPX* from *APX2* regulation (Galvez-Valdivieso *et al.*, 2009).

Transcript levels of both sugar markers (β -AMY and APL3, Baier *et al.*, 2004) increased in high light, more so in N→H plants which is not surprising since the L→H plants still had to replete their carbohydrate pools after the extended period of starvation. The stronger up-regulation of sugar markers in N→H plants than in L→H plants appears to exclude the possibility that sugars control wwc expression since L→H plants revealed the stronger wwc transcript responses compared with N→H plants.

Intersystem electron transport- and plastoquinone-dependent signalling regulates plastocyanin transcript and protein levels (Pfannschmidt *et al.*, 2001; Schütze *et al.*, 2008) where a reduced PQ pool triggers up-regulation and an oxidized PQ pool a down-regulation of *PETE* transcripts. *PETE2* was unresponsive to the N→H- as well as the L→H-light treatment. It is likely that the decrease in photosynthetic yield and activation of photoinhibition efficiently suppress PQ-dependent signalling at least 6 h after transfer to H-light. *AOX1A* is the dominant alternative oxidase in leaves and known to respond to H-light and is likely to be dependent on the redox state of the mitochondrial respiratory chain as indicated by correlation with the ubiquinone reduction state. *AOX2* transcript is mostly unresponsive to environmental changes (Yoshida and Noguchi, 2009). In line with this study, *AOX2* transcript levels were unaffected by the N→H- as well as the L→H-light transfer. For the time being, *AOX1A* transcript amounts may be taken as an indicator of the NAD(P)H reduction state. Interestingly, the reduction states of both the GSH/GSSG and the AA/DHA couples, were not significantly changed in L→H- or N→H plants, suggesting that plants have sufficient reductive power at that point.

The lipid markers *LOX3* and *PPTE* (Goda *et al.*, 2008) revealed the strongest up-regulation in both treatments indicating that lipid peroxidation products should be investigated as potential signals in the H-light response in more detail. In this context it will be important to explore the light-dependent dynamics of enzymatically versus ROS-dependently produced lipid peroxidation products such as hydroxy fatty acids, 12-oxophytodienoic acid, and jasmonic acid as potential signalling compounds.

Adjustment loops elicit transcript responses prior to protein deficit

Protein contents provide a rough estimate of plasmatic volume. Assuming a protein content of 25% (w/v), typical for plasmatic compartments like the cytosol and stroma (cf. Dietz and Heilos, 1990), glutathione concentrations were similar in L- and N-plants. L-plants accumulated higher protein amounts of wwc enzymes despite a lack of metabolic requirement under these low-light conditions. It may be speculated, that this prepares for future episodes with higher light. The strong up-regulation of wwc transcripts in the presence of high enzyme concentrations in N→H-plants or above normal levels in L→H-plants and the unexpectedly low generation of ROS in L-plants suggest the existence of regulatory loops independent of the specific activity of the wwc. The combinatorial application of ABA, DCMU, and light showed the predominance of DCMU-linked suppression of *sAPX* transcripts even in the presence of high light. As an inhibitor of the plastoquinone binding site of PSII, DCMU is reported to elicit $^1\text{O}_2$ signalling (Flors *et al.*, 2006) and to decrease photosynthetic metabolism, but hardly affects respiratory O_2 uptake (Reddy *et al.*, 1991). The DCMU-dependent decrease in *sAPX* accumulation suggests that $^1\text{O}_2$, either alone or in combination with other photosynthetic signals, is not involved in *sAPX* up-regulation. ABA even suppressed the H-light-dependent *sAPX* accumulation, further supporting the conclusion that ABA is unlikely to be involved in the regulation of the transcripts of the wwc enzymes. Also plastoquinone-dependent signalling seems not to play a major role based on two observations. Expression of the typical marker transcript for PQ redox signalling *PETE2* (Pfannschmidt *et al.*, 2001) hardly responds in N→H- and L→H-plants, while LHCII transcript levels dropped as expected if growth light increases. In addition, the wwc transcripts were up-regulated, especially under low light, in the *stin7* mutant which is defective in PQ-dependent signalling (Pesaresi *et al.*, 2009). On the other hand, the down-regulation of *sAPX* in the presence of DCMU is in line with a contribution of photosynthetic electron pressure to wwc transcript regulation. According to previous results it is suggested that redox cues downstream of photosystem I need to be considered as a possible origin of signalling in this context (Rintamäki *et al.*, 2000; Bräutigam *et al.*, 2009). The feedback loops may involve metabolites, redox signals, and lipid-derived signalling. Sucrose feeding influences, for example, ADP-pyrophosphorylase (AGPS) or β -amylase

transcript amounts (Sokolov *et al.*, 1998; Baier *et al.*, 2004; Muller *et al.*, 2005). The strong up-regulation of marker genes for sugar- and metabolite-responsive signal transmissions implies that these signals may act as possible retrograde signalling pathways. However, it is unlikely that these pathways control wwc. Overall, the genes of the wwc enzyme do not show a uniform response and appear to be controlled by multiple cross-talking signalling pathways.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Total protein and RNA contents of N-, L-, N→H-, and L→H-plants.

Supplementary Fig. S2. Protein quantification in immunoblots in L-, N-, L→H-, and N→H-leaves at 0, 6, and 24 h.

Supplementary Fig. S3. Transcript quantification of water–water cycle enzymes in mutants and wild type by semi-quantitative RT-PCR in L-, N-, L→H-, and N→H-leaves at 0, 6, and 24 h.

Supplementary Table S1. Primers for semi-quantitative and quantitative RT-PCR.

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